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Tuberculosis bacillary load, an early marker of disease severity and treatment response: the utility of tuberculosis Molecular Bacterial Load Assay

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Tuberculosis bacillary load, an early marker of disease severity and treatment response: the utility of tuberculosis Molecular Bacterial Load Assay

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ABSTRACT

In this comparative biomarker study, we analysed 1768 serial sputum samples from 178 patients from 4 sites in South-East Africa. We show that tuberculosis Molecular Bacterial Load Assay (TB-MBLA) reduces time-to-TB-bacillary-load-result from days/weeks by culture to hours and detects early patient treatment response. By day-14 of treatment, 5% of patients had cleared bacillary load to zero, rising to 58% by 12th week of treatment. Fall in bacillary load correlated with MGIT culture time-to-positivity (Spearmans r=-0.51, 95% CI (-0.56 to -0.46), p<0.0001). Patients with high pre-treatment bacillary burdens (above the cohort bacillary load average of 5.5$\log_{10}$eCFU/ml) were less likely to convert-to-negative by 8th week of treatment than those with a low burden (below cohort bacillary load average), p=0.0005, HR 3.1, 95% CI (1.6-5.6) irrespective of treatment regimen. TB-MBLA distinguished the bactericidal effect of regimens revealing the moxifloxacin - 20mg rifampicin regimen produced a shorter time to bacillary clearance compared to standard-of-care regimen, p=0.008, HR 2.9, 95% CI (1.3-6.7). Our data show that the TB-MBLA could inform clinical decision making rapidly and expedite drug tuberculosis clinical trials.

INTRODUCTION

Tuberculosis is among the top 10 causes of death globally and has overtaken HIV/AIDS as a leading cause of death from a single infectious agent. An estimated 10 million fell ill of TB in 2018 and approximately 1.5 million died[1]. Treatment is difficult, requiring a combination of four drugs and, depending on whether TB are drug-susceptible or -resistant, takes as long as six or twelve months to treat. To help health care workers managing TB make better decisions we need to develop effective methods to monitor the response to treatment. Such a marker of treatment response would reduce costs associated with prolonged care of patients who otherwise already converted to negative, and in tuberculosis clinical trials by speeding up drug development[2,3]. Effective methods would help identify those who are failing on treatment and require a change in therapy, or do not convert to negative early on in treatment and may require prolonged treatment period[4].

A high bacterial burden is known to be associated with a poor outcome but measuring it is difficult. A fall in bacterial burden is the most relevant marker of treatment response currently available, but culture is technically difficult to standardize and not all viable *Mycobacterium tuberculosis* (Mtb) bacilli are detected[5,6]. Alternative more rapid methods include mycobacterial DNA-detection assays but prolonged DNA survival in the host after organisms have been killed precludes their use for treatment monitoring[7]. The TB-MBLA, a real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) uses more abundant 16S-rRNA as a target, increasing its sensitivity to accurately quantify Mtb viable bacillary load to as low as 10 CFU/ml and over many weeks of treatment[8,9]. Treatment response (change in bacillary load) can be detected as early as 3 days on treatment[8]. Here we report the first multi-centre evaluation of TB-MBLA in comparison to standard-of-care culture methods for measuring TB treatment response in three high-burden sub-Saharan African countries.

METHODS

A total of 213 therapy naïve pulmonary tuberculosis patients were enrolled for treatment response assessment at four sites in Tanzania (site 1 & 2), Mozambique (site 3) and Malawi (site 4). The Tanzanian sites were part of the PanACEA MAMS-TB clinical trial (NCT01785186) and were on different regimens, all other participants were on standard of care HRZE/HR. Serial patient sputum samples were collected weekly before and during treatment. Treatment response was measured over 12-week treatment period as change bacillary (bacterial) load (estimated (e) CFU/ml) measured by TB-MBLA, compared to culture positivity and/or culture time-to-positivity (TTP) by Lowenstein-Jensen (LJ) medium and the Mycobacterial Growth Indicator Tube (MGIT) culture.

Spearman rank correlation (r) was used to assess relationship between change in bacillary load and TTP in response anti-TB therapy. Rate of sputum clearance for each patient was calculated as the SLOPE of the patient bacillary load and TTP over time using Microsoft Excel 2016 (y=bacillary load, x=time on treatment). Patient "conversion" was defined as a change from 'positive' to 'negative (two consecutive negative results)' without subsequent reversion to 'positive' before the end of 12-week follow-up. The day of conversion was defined as the midpoint between last positive and first definite negative result. A negative result for TB-MBLA was considered at RT-qPCR quantification cycle above 30 (zero bacillary load). Patients with above the mean cohort bacterial load at baseline were categorised as "high bacterial load" and "low bacterial load" for those below the mean. Hazard ratio for conversion to negative by patients with high- versus low-baseline bacillary load, HIV+ or HIV- and/or treated with experimental regimens compared to standard-of-care (control) regimen was examined using Mantel-Cox and Gehan-Breslow-Wilcoxon tests. One-way analysis of variance (ANOVA) was applied to examine inter-site variance in TB-MBLA performance. In all analyses p values were considered significant at $p<0.05$. All statistical tests apart from SLOPE calculations were performed in Graphpad Prism v.6.

Ethical approval for the study was obtained from relevant ethics committees in Tanzania (NIMR/HQ/R.8c/242), Mozambique (147/CNBS/14) and Malawi (P.08/13/1448) (detailed methods on line file 1)

RESULTS

Of the 213 patients, 178 (83·6%) patients completed 12-week follow-up were included in the analysis. Males, 128 (71.9%) constituted the majority of cases, 47 (26.4%) were HIV positive and the median age of the whole study group was 33 years (IQR: 27-40 years). In 164 (92%) patients had susceptible TB (more details of patient characteristics online supplement figure 1). The rate of bacillary load clearance was high, ≥ 1 $\log_{10}$eCFU/ml/week in the first two weeks of therapy. As a result, 5% (9) patients had converted to zero bacillary load by day 14 of treatment. This number rose to 40% (71) and over half the cohort, 58.4% (104) by 8th and 12th week of treatment. Fall in bacillary load was inversely correlated with increase in TTP (Spearman r = -0.51, 95% CI (-0.56 to -0.46), $p<0.0001$). The mean bacillary load at baseline (±standard deviation) was 5.5±1.3

declining to 1.7±1.4 at week eight and 0.9±1.2 log_{10} eCFU/ml at week 12. MGIT culture TTP, median (range) at baseline was 5 (2-8) days, increasing to 21 (16-26) and 25(14-36) days by week 8 and 12 respectively (Figure 1).

While MGIT time-to-result increased with fall in bacterial load, from 5 to 25 days), TB-MBLA time-to-result was the same, 5±1h regardless of the patient bacillary load. Patients with high baseline bacterial load were less likely to convert to negative by 8th and 12th week of treatment, p=0.0005, HR 3.1, 95% CI (1.6-5.6) and p=0.0008, HR 2.0, 95% CI (1.3-3.1) irrespective of treatment regimen and rate of sputum bacillary clearance in the first two weeks of treatment. Among patient characteristics, only HIV co-infection reduced the chance to clear bacillary load by 12th week of treatment p=0.02, HR 2.1 95% CI (1.2-3.7).

TB-MBLA showed that compared to control regimen, the rifampicin (RIF) 20mg/kg plus moxifloxacin and RIF 35mg/kg regimens had significantly higher bactericidal effect, 89% and 56% conversion to negative by week 12 of treatment (Table 1). The RIF 35mg/kg regimen result is consistent with culture in the MAMS study[10], even though our study had additional HRZE patients from outside the study. TB-MBLA results were reproducible in different laboratory settings, ANOVA p>0.05 and not affected by contamination (Online supplement figures 2 and 3 and table 1)

	Control (RIFHZE)	$RIF_{20}MHZ$	$RIF_{35}HZE$	$RIF_{20}QHZ$	RIFQHZ
Number analysed	32	18	17	16	16
Number converted by day 56 (week 8)	9 (28%)	11 (61%)	7 (41%)	6 (38%)	3 (19%)
Number converted by day 84 (week 12)	18 (56%)	16 (89%)	10 (59%)	9 (56%)	9 (56%)
Median time to conversion to negative (IQR)	77 (56-84)	56 (42-77)	70 (35-74)	74 (35-84)	70 (56-84)
Log-rank (Mantel-Cox) test					
Hazard ratio (95% CI)	--	2.9 (1.3-6.7)	2.3 (1.0-5.2)	1.2 (0.5-2.9)	1.1 (0.5-2.5)
P value	--	0.008	0.049	0.663	0.853
Gehan-Breslow-Wilcoxon test					
Hazard ratio (95% CI)	--	2.3(1.4-6.3)	1.9 (1.1-4.9)	1.2 (0.5-2.8)	1.1 (0.5-2.4)
P value	--	0.008	0.063	0.632	0.791

Table 1 MBLA assessment of the clinical trial regimens compared to standard regimen. The $RIF_{20}MHZ$ and $RIF_{35}HZE$ treated cases had significantly shorter conversion time to negative than those treated with standard regimen. RIF=Rifampicin, M=Moxifloxacin, H=Isoniazid, Z=Pyrazinamide, E=Ethambutol and Q=SQ09.

DISCUSSION

Confirming the diagnosis and determining whether patient is responding to the prescribed therapy is crucial for healthcare workers managing tuberculosis patients. In this study we have shown that TB-MBLA gives rapid bacillary load count, which responds to therapy in a pattern consistent with liquid culture, takes shorter time-to-result and is reproducible in different laboratory settings. Early determination of treatment response facilitates questions on adherence and/or drug resistance, and identifying patients at risk of failing treatment particularly those with high bacillary load. In addition, TB-MBLA simplifies evaluation of the impact of comorbidities such as HIV on bacillary load clearance during treatment. Compared to the current treatment monitoring methods, microscopy which is less sensitive and cannot distinguish viable from dead bacilli and culture with long time-to-result, we believe TB-MBLA has potentially higher utility for informing clinical decisions on individual patient management and facilitating rapid evaluation of anti-TB drugs in clinical trials.

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Author contributions

Study design: SHG, TDM, KO, WS, IH, AR, NH; MH, MB, GSK; GM, MB Literature search: SHG, IH, TDM, Training: WS, SHG, KO, DE, IH, TDM, MZ, Data collection: KA, DK, BM, MK, AM, ECCF, GSK, MK, EK; NET, NB, SV, IJ, ECWF, Data analysis: SHG, RB, WS, DS; Data interpretation: SHG, DE, WS, RB; Drafting the paper: WS, RB, DS, SHG; Figures and tables: WS, DE; Manuscript reviewing: All authors.

Conflict-of-interest statement

Authors declare no conflict of interest.

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Figure legends

Figure 1. Bacteriological response to treatment measured by the TB-MBLA (A) and by MGIT (B) at four study sites. The curves indicate the average bacillary load reduction mirroring increase in culture time-to-positivity by all patients over a 12-week treatment period. Dots represent individual patients at each site. Data points are widely spread in culture, which imply high culture result variability in late stages of treatment. Black curves with thick black dots represent Clinical trial Site 1 and 2 cases, Red curves and red diamond clear symbols represent site 3 cases and Blue curves with black clear dots represent Site 4 cases (MGIT culture stopped at week 8).

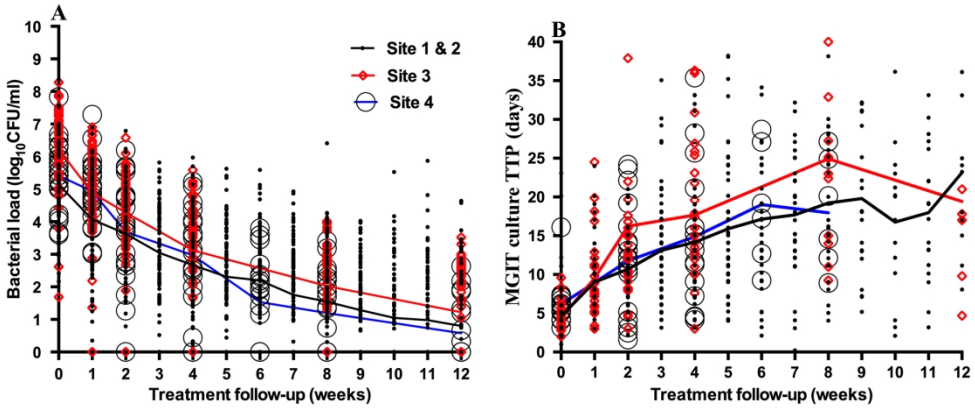
Supplementary material

Online supplement Figure 1. The study flow diagram. Patients who died earlier on in the study, lost to follow-up, missing baseline sample, missing ≥2 treatment visit results and/or had no data at all were excluded from the analysis, leaving 178 patients.

Online supplement Figure 2. The reproducibility of TB-MBLA in different laboratory settings. A) the results of high BCG panel (n=24 per site), B) results of low concentration BCG panel (n=24 per site), and C) the extraction (internal control retrieved from patient samples as marker of extraction efficiency (n=1633 across the 4 sites). Each point represents an extracted sample and the red line is the median of the log eCFU/ml of the spiked extraction control.

Online supplement Figure 3. A comparison of MGIT culture and TB-MBLA results. Orange and green bars are cases with definitive MBLA positive or negative result but indeterminate MGIT culture result due to contamination that grew in number in late stages of treatment.

Online supplement table 1: The MGIT and LJ culture results showing the increasing contamination rates as patients progressed on treatment. There were more contaminations in MGIT than LJ.



Treatment response over the 12-week period

175x77mm (300 x 300 DPI)

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Key messages

What is the key question?
What is the utility of using TB-MBLA to monitor TB treatment response compared to standard culture methods?

What is the bottom line?
The TB-MBLA measured bacillary load falls in a manner consistent with increasing culture-time-positivity as the patient responds to anti-TB therapy but unlike culture, the TB-MBLA quantitative result is available in hours instead of days or weeks, unhampered by contamination and reproducible in diverse settings.

Why read on?
Fourteen days into treatment, 5% of the patients had converted to negative with zero bacillary load while over half of all the patients had done the same by 12th week of treatment, revealing that most TB patients convert to negative early in treatment and may not require prolonged stay on anti-TB therapy. These results show the power of a rapid bacillary load result delivered by TB-MBLA to inform individual treatment decisions, early detection of poor treatment responders who need longer time on therapy and/or change of course for those on ineffective therapy.

INTRODUCTION

Tuberculosis (TB) is among the top 10 causes of death globally and has taken over HIV/AIDS as a leading cause of death from a single infectious agent. An estimated 10 million suffered TB in 2018 and approximately 1.5 million died[1]. Treatment is difficult requiring combination of four drugs and depending on the form of TB, susceptible or resistant takes as long as six or twelve months to treat. The number of patients who require retreatment is high and 18% of these account for the half million drug-resistant tuberculosis cases[1]. To help health care workers managing tuberculosis (TB) make better decisions we need to develop effective methods to monitor the response to treatment[2]. Such a marker of treatment response would reduce costs in tuberculosis clinical trials and speed drug development[3,4]. Effective methods would help identify those who convert to negative early on in treatment and don't require prolonged treatment period and vice versa for those failing on treatment[5].

Definitive diagnosis of tuberculosis depends on the culture of *Mycobacterium tuberculosis*, the current gold standard. For most TB control programs there is, however, limited availability of culture due to the cost of high containment laboratories, staff training and reagent supply. With a culture-based approach results are available too late to inform timely clinical decision making. Factors such as specimen transport, type of media, and chemical decontamination to eliminate

non-mycobacterial flora compromise the consistency of culture. In addition, culture time-to-result is long and depends on the level patient TB burden. This means that low burden patients wait longer for results than their high burden counterparts. Sputum culture conversion is only weakly predictive of long-term patient outcome for regimens but not individuals limiting its utility to individual and clinical trial management[6]. Smear microscopy, an approved routine care treatment monitoring is less sensitive and cannot distinguish between viable and dead bacilli[7].

Enumeration of viable mycobacteria during therapy is probably the most accurate biomarker of treatment response currently available, but culture is technically difficult to standardise and not all viable Mtb bacilli are detected[8,9]. Alternative more rapid methods include mycobacterial DNA-detection assays but prolonged DNA survival in the host after organisms have been killed precludes their use for treatment monitoring[10]. Messenger RNA (mRNA) targets, with a shorter half-life than DNA including *fbpB* antigen 85B, *hspX*, and *icl*, have been tested as treatment response biomarkers. Their decline during treatment mirrors traditional *M. tuberculosis* colony counting[11–13] but, as mRNA exists at low concentrations, mRNA based assays reach their limit of detection rapidly when patients are still TB culture positive[12,13]. These assays are valuable for following early responses, but less effective for monitoring over a longer period of treatment.

The tuberculosis Molecular Bacterial Load Assay (TB-MBLA), a real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) uses more abundant 16S-rRNA as a target and can accurately quantify *M. tuberculosis* viable bacillary load over many weeks of treatment[14] and could replace solid culture[15]. Here we report the first multi-centre evaluation of the TB-MBLA in comparison to standard methods in three high-burden countries in sub-Saharan Africa and show that it assesses disease severity and patients' response to treatment in real-time to inform clinical decision and is reproducible across different laboratory settings.

METHODS

Sites, patients and sample schedule

Tanzania (Site 1 and 2): Patients were recruited from the PanACEA Multi-Arm Multistage (MAMS) TB trial sites, Kibong'oto National Tuberculosis Hospital-Kilimanjaro Clinical Research Institute (KCRI) and National Institute of Medical Research at Mbeya Medical Research Centre (NIMR-MMRC)[16]. Tuberculosis was confirmed by sputum smear microscopy or GeneXpert. Early morning and spot sputum samples were collected at enrolment and weekly for 12 weeks. Culture was by Lowenstein Jensen (LJ) media and in the Mycobacterial Growth Indicator Tube (MGIT) liquid culture system and spot sputa were used for TB-MBLA on site. If insufficient material was available, the specimen was used for MGIT liquid culture.

Mozambique (Site 3): Sputum smear and GeneXpert MTB/RIF positive patients, irrespective of HIV status were recruited from the Maputo Tuberculosis Trial Unit (MaTuTU). MDR-TB was not an exclusion criterion. Patients received WHO recommended treatment for susceptible or MDRTB as appropriate. Early morning and spot sputum samples were collected at enrolment and then one, two four, eight and 12 weeks.

Malawi (Site 4): Sputum smear or GeneXpert MTB/RIF positive patients were recruited from the TB clinic, Queen Elizabeth Central Hospital, College of Medicine, Blantyre. All patients except

two retreatment cases received WHO recommended standard treatment for susceptible disease. Early morning and spot sputa were collected at enrolment, then 2,4,6,8 and 12 weeks.

Ethics

Consent for collection and laboratory evaluation of samples from MAMS-TB study was obtained from National Institute of Medical Research (NIMR) for the Tanzanian sites, NIMR/HQ/R.8c/242. Approval was also obtained from Instituto Nacional de Saude (INS) Institutional Review Board and National ethics committee for the Mozambique site, 147/CNBS/14 and College of Medicine Research Ethics Committee (COMREC) University of Malawi for the Malawian site, P.08/13/1448.

Molecular and Microbiological methods

Culture based measures: All sputum samples were tested by quantitative microscopy, culture on Lowenstein-Jensen medium and MGIT as previously described[12,13]. Positive MGIT cultures were tested for acid fast bacilli (AFB) positivity and contamination using Ziehl Neelsen (ZN) staining and blood agar respectively. AFB and Antigen MPT64 negative but blood agar positive cultures were considered contaminated (indeterminate). AFB and MPT64 positivity confirmed presence of *Mycobacterium tuberculosis* (Mtb) whilst blood agar positivity result within 24h confirmed presence of contaminating flora.

Tuberculosis Molecular Bacterial Load Assay (TB-MBLA):

Sputum aliquots (1ml volume) were preserved for TB-MBLA by diluting 1:4 in 50% guanidine thiocyanate (GTC) w/w, 0·1M Tris HCl pH 7·5 and 1% β-mercaptoethanol v/v then stored at -80°C until testing. Testing was done in batches with majority of samples tested between 1-4 weeks from collection. Prior to RNA extraction, samples were thawed at room temperature and internal control (100µl) added. Mtb cells were harvested by centrifugation 3000 *g* for 30 minutes and total RNA extracted using the RNA Pro Blue kit (MP Biomedicals following manufacturer's instructions[11]. Reverse transcriptase qPCR of the RNA was performed as described previously using RotorGene 5plex platform (Qiagen)[11]. Primers and Taqman dual labelled probes targeting *M. tuberculosis* and the internal control were procured from Eurofin Genomics, Germany (Note: the oligos have been incorporated into the TB-MBLA kit and cannot be published). Standard curve was used to convert quantification cycles (Cq) into bacterial (bacillary) load (estimated colony forming units per ml (eCFU/ml) and log transformed at a factor of $\log_{10}$. A high (10^7 CFU/ml) and low (~~10^3~~-10^4 CFU/ml) positive control (BCG NCTC 5692) and negative control of RNase free molecular grade water were included in each assay run. The limit of detection of TB-MBLA was considered at 30 Cq, equivalent to 10 eCFU/ml bacillary load.

Quality assurance and applicability of the MBLA assay in different laboratory environments:

To verify the quality assurance, reproducibility and applicability of the different settings we analysed how participating laboratories processed the externally supplied standard BCG positive control panels. Two panels of material containing either a high concentration, 10^7 CFU/ml and low concentration, 10^4 CFU/ml (n=24) were supplied by the University of St Andrews TB laboratory to all participating sites. Each laboratory independently conducted RNA extraction and qPCR and analysis.

Analysis

Correlation of TB-MBLA and MGIT was determined using Spearman rank correlation (r). Treatment response was defined as a change in bacterial load measured by TB-MBLA, time to culture positivity (TTP) by liquid MGIT and/or positivity/negativity of TB-MBLA, MGIT and LJ. Rate of sputum clearance was calculated as the SLOPE of the patient bacillary load and TTP treatment response curves (y=bacillary load and x=time on treatment) using Microsoft Excel 2016. Patient "conversion" was defined as a change from 'positive' to 'two consecutive negatives' without subsequent reversion to 'positive' before the end of follow-up. "Non-conversion" was defined as persistent or recurrently positive samples by the final visit on day 56 and 84 for 2-months and 3-months outcome respectively. The day of conversion was defined as a midpoint between last positive and first definite negative result. One-way analysis of variance (ANOVA) was applied to examine inter-site variance in TB-MBLA performance. In all analyses p values were considered significant at $p<0.05$. Apart from SLOPE, all statistical analyses were performed with GraphPad Prism v.6.

RESULTS

TB-MBLA measured bacillary load inversely correlates with culture time to positivity in TB patients

Patients and samples: A total of 213 anti-tuberculosis treatment naïve patients were enrolled for treatment response monitoring using TB-MBLA in comparison with culture at four study sites. Out of the 2103 cases, 101 were under the MAMS clinical trial sites in Tanzania[16] whereas 112 cases were enrolled from routine practice sites in Mozambique and Malawi. 178 (83·6%) completed the 12-week treatment follow-up and were included into the analysis: Tanzania 100 (55·9%), Mozambique 58 (32.8%), and Malawi 20 (11.3%) (supplementary figure 1). Males, 128 (71.9%) constituted the majority of cases, 47 (26.4%) were HIV positive and the median age of the whole study group was 33 years (IQR: 27-40 years). A total of 1768 serial samples were tested over a period of 12 weeks of treatment follow-up. In 164 (92%) patients, the organisms were fully susceptible while 14 patients had drug resistance: 7 (4%) were pyrazinamide monoresistant, 3 (1.7%) were polyresistant to isoniazid and pyrazinamide while 4 (2.5%) were multidrug resistant TB (MDR-TB) and managed with MDR regimen including kanamycin, levofloxacin, ethionamide, cycloserine, and pyrazinamide.

Treatment response and implication of high pre-treatment bacterial burden: Over the 12 weeks of treatment there was a steady decline in bacillary load at a rate of sputum clearance, $0.4\log_{10}$eCFU/ml/week corresponding to 1.1 days/week MGIT culture time to a (TTP). The rate of sputum bacillary load clearance was faster $>1\log_{10}$eCFU/ml/week in the first two weeks of treatment. As a result, 5% (9) patients had zero bacillary load by week two of treatment. The number of patients converting to zero bacterial load rose to 40% (71) and 58.4% (104) by 8^{th} and 12^{th} week of treatment. Fall in bacillary load was inversely correlated with increase in TTP, Spearman r = -0.51, 95% CI (-0.56 to -0.46), *p<0.0001*. The mean bacillary load (±standard

deviation) at baseline, 5.·5±1.·3 declining to 1.·7±1.·4 at week eight and 0.·9±1.·2 $\log_{10}$ eCFU/ml at week 12. MGIT culture TTP, median (range) at baseline was 5 (2-8) days, increasing to 21 (16-26) and 25(14-36) days by week 8 and 12 respectively. Using logistic regression, we found that patients with high baseline bacterial load (≥6$\log_{10}$eCFU/ml) were less likely to convert to negative by 8th week of treatment, OR 1.5, p=0.002 and this was independent of their rate of clearance in the first 2 weeks of treatment (figure 1 main paper).

Despite the higher number of males in the study, their mean bacterial load 5.6 ± 0.1 and time-to-conversion 50.8± 0.9 days in the first 2 months of treatment did not differ from that of females, 5.4 ± 0.2, 47.1±2.0 days, Unpaired t test, p=0.37 and 0.06 respectively.

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Time to result: The TB-MBLA result was available in 5±1h from the time the sample was received at the laboratory and was independent of the patient bacillary load. In contrast, the time-to-culture result increased with decline in bacterial load, starting from 5 days at baseline to 25 days at 12 weeks of treatment.

TB-MBLA concurred with culture on distinguishing the bactericidal effect anti-TB regimens

We analysed the performance of TB-MBLA for distinguishing the bactericidal effect of four experimental regimens compared to the control on a cohort of 100 MAMS trial patients in Tanzania[16]. The four regimens were different combinations of high dose rifampicin (RIF) 20mg/kg and 35mg/kg, moxifloxacin (M), and SQ109 compared to standard-of-care drugs, RIF, Isoniazid (H), pyrazinamide (Z), and ethambutol (E). Percent conversion to negative (two consecutive zero bacterial load results without reversion to positive) was calculated. Conversion in $RIF_{20}MHZ$, 89% and $RIF_{35}HZE$, 59% were higher than the control RIFHZE, 56% at week 12 of treatment. Using Mantel-Cox and Gehan-Breslow-Wilcoxon tests we asked whether time to conversion to negative was different between the experimental regimens and standard-of-care (control) regimen. $RIF_{20}MHZ$ had significantly shorter time-to-negative conversion, p=0.008 for both tests whilst $RIF_{35}HZE$ was weakly significant, p=0.049 in the Mantel-Cox test and trend, p=0.06 for the Gehan-Breslow-Wilcoxon (Table 1 main paper)

TB-MBLA is robust and reproducible in different laboratory settings

A TB monitoring test for clinical use needs to be performed consistently in different clinical laboratories. To achieve this, we analysed variance of the qPCR results of standard BCG panels (high concentration, log7- and low concentration, log4- CFU/ml) and extraction (internal) control, log5 CFU per patient sample independently processed at the four study sites. The measured bacterial load from standard BCG panels were consistent between sites, median (IQR) 7.1(7.0-7.6), 7.1 (6.9-7.6), 7.4 (6.9-7.8), 7.4 (7.2-7.7) $\log_{10}$ eCFU/ml, coefficient of variation (CV), 4.87%, 4.39%, 7.26%, 4.96% for the high concentration panel (supplementary figure 2A) and 3.4 (3.1-3.7), 3.5 (3.1-4.1), 3.3 (3.0-3.8), 3.6 (3.3-3.8), CV 15.80%, 18.70%, 15.36%, 12.43% for low

concentration panel (supplementary figure 2B) for sites 1, 2, 3 and 4 respectively. Similarly, the internal control was consistently retrieved, median (IQR) 4.4 (3.5-5.1), 3.8 (3.4-4.3), 4.2 (3.7-4.6), 3.9 (3.1-4.5) and CV 19.10%, 16.42%, 13.75%, 19.15% for the four sites respectively. Taken together, the average retrieval of internal control was 4.1±0.7 log_{10}eCFU/ml, which is equivalent to 82% (4.1/5.0 log_{10}eCFU/ml) RNA extraction efficiency (supplementary figure 2C). The ANOVA probability value was >0.05 between sites for both positive and internal extraction control.

TB-MBLA is not affected by contaminating flora in patient sputum

At baseline, 171 (98%) patients generated quantitative TB-MBLA eCFU/ml and MGIT TTP results. The number of dually positive samples decreased steadily as the number of positives declined and by week twelve only 16 (10.7%) were positive by both assays. A total of 232 (14.8%) samples were MBLA positive - MGIT contaminated including 32 (21.3%) from week 12. No patients were both negative by MBLA and MGIT at baseline, but this figure rose to 20 (13.3%) by week 12. "MBLA negative, MGIT contaminated" was found in 195 (12.4%) specimens, including 61 (40.7%) at week 12. The rate of culture contamination increased with time on treatment resulting in fewer MGIT culture samples available for TTP analysis, 19 (11%) by week 12 of treatment. In total 427 (27.2%) samples with a definite "positive" or "negative" result on MBLA were lost to contamination by MGIT. Only 70 (4.5%) samples were MBLA negative and MGIT TTP positive (putative MBLA false negative). In comparison, 103 (6.57%) were MBLA eCFU/ml positive but MGIT negative (putative MGIT false negative) (supplementary Figure 3).

Solid (LJ) cultures only were less prone to contamination than MGIT at all time-points but less sensitive than MGIT and TB-MBLA (supplementary table 1).

		Number of samples (%) in weeks							
	Week	**0**	**1-2**	**3-4**	**5-6**	**7-8**	**9-10**	**11-12**	**Total**
MGIT	Positive	171 (96)	278 (91)	197 (81)	117 (56)	92 (39)	42 (25)	40 (17)	937 (60)
	Negative	2 (1)	11 (4)	10 (4)	22 (11)	51 (22)	44 (26)	63 (26)	203 (13)
	Contaminated	1 (1)	15 (5)	36 (15)	70 (33)	94 (40)	84 (49)	137 (57)	427 (27)
LJ	Positive	115 (77)	186 (74)	112 (52)	71 (38)	37 (23)	19 (10)	10 (5)	554 (40)
	Negative	27 (18)	60 (24)	95 (44)	105 (56)	115 (71)	160 (86)	166 (90)	768 (56)
	Contaminated	6 (4)	4 (2)	7 (3)	10 (5)	9 (6)	7 (4)	8 (4)	51 (4)

Supplementary table 1: The MGIT and LJ culture results showing the increasing contamination rates as patients progressed on treatment. There were more contaminations in MGIT than LJ.

DISCUSSION

The most important questions facing healthcare workers managing patients with tuberculosis is to confirm the diagnosis and to determine whether the patient is responding to the prescribed therapy. Failure to respond may be due to multiple factors including poor adherence, poor absorption of drugs, or a drug-resistant infecting organism. Bacillary load is an established biomarker for disease severity and treatment outcome[17–19], yet the current diagnostic methodologies do not provide the information to address crucial clinical questions in a meaningful time-frame. In this paper, we have shown that TB-MBLA gives rapid bacillary load count, which responds to therapy in a pattern consistent to liquid culture, is reproducible in different laboratory settings, more sensitive than culture and reduces time-to-result from days or weeks to hours. We believe these properties increase TB-MBLA's utility for informing clinical decisions on individual patient management drugs and facilitating rapid evaluation of anti-TB drugs in clinical trials. From the perspective of the treating healthcare worker, the TB-MBLA provides a readily understandable result, a number of viable bacteria compared to time-to-positivity.

When plotted as a patient journey, the decline in bacillary load is an inverse image of the MGIT TTP results over the first three months of treatment[14]. A recent study has further confirmed this, demonstrating that TB-MBLA is more consistent to culture in detecting viable bacilli than Xpert MTB/RIF and smear microscopy[20]. This suggests that they are monitoring broadly similar measures in the viable count. In the first few weeks following the initiation of treatment, it is important to understand whether the patient is responding. Radiological appearances do not resolve in a timely or reproducible way[21]. MGIT is a semi-quantitative measure of viable count as the patient responds to therapy, as the time to a positive culture result increases, the time taken to report a useful result increase proportionately. Direct quantification of *M. tuberculosis* RNA in the sample means that the level of patient bacterial burden does not influence the TB-MBLA time-to-result in the laboratory. In contrast time to result in culture depends on patient bacterial burden of which low burden patients wait longer to receive their results.

Like culture, molecular bacillary load clearance is biphasic, faster in the first 14 days of treatment and slow in the subsequent weeks of treatment follow-up. This may be explained by the hypothesis that anti-TB drugs work by fast clearance of the actively growing bacilli and slowly for the dormant (persister) population[22]. High baseline bacterial load was predictive of failure to convert to negative at 8 weeks of treatment, consistent with previous findings with culture [23–25]. All the 4 MDRTB patients were among those who did not convert to negative by week 8, however, this was too small a number to make any comparative modelling of the impact of MDRTB on treatment response measured by TB-MBLA. Among the patient characteristics, only HIV coinfection reduced the chance to convert to negative (zero bacillary load) by 12th week of treatment. This observation contrasts earlier studies where bacterial load clearance was not different between HIV-infected and HIV-uninfected patients[26,27], and thus requires further investigation.

TB-MBLA largely concurred with MGIT culture on the shorter time to conversion of the clinical trial patients treated with high dose rifampicin regimen. However, in this cohort of 100 Tanzanian patients, the moxifloxacin-20mg/kg RIF regimen gave a significantly shorter time to conversion compared to HZE-35mg/kg RIF regimen that emerged the most effective regimen according to the

MAMS study findings. The MAMS results were based on culture conversion of the whole MAMS study cases from Tanzania (part of this study) and South Africa[16]. The better performance of the moxifloxacin regimen may be explained by the reported rapid bactericidal effect in the early weeks of treatment but poor sterilizing effect in the continuation phase of treatment[28]. The limitation of this comparison is that the two tests (TB-MBLA and MGIT culture) were not performed on equal number of cases and for the same length of treatment follow-up. Future larger trials involving both tests will give a more accurate comparison of their ability to assess the bactericidal effect of anti-TB therapy in relation to treatment outcomes.

To be useful and accessible to a wider community, it is crucial that the diagnostic test is robust and its results are reproducible. This paper shows the TB-MBLA test results are reproducible in different laboratory settings. We have shown previously that the tuberculosis molecular bacterial load assay is species specific [14,29,30], providing the healthcare worker with both confirmation of the tuberculosis diagnosis and response to therapy. The use of the extraction (internal) control in TB-MBLA enables normalisation of results based on the amount retrieved compared to the amount that was spiked in prior to extraction process. The 82% extraction efficiency is consistent to the one reported by Ratnam et al at centrifugation force, 3005 – 3895g[31]. The loss in extraction could be attributed to the poor sedimentation of mycobacteria during the initial centrifugation step as reported by several studies[31–34].

Despite being a gold standard, culture results are compromised by growth of non-TB flora in sputum. Attempts to remove these flora using sodium hydroxide and/or suppress them by antibiotics in liquid culture, malachite green in Lowenstein Jensen reduce the viable bacterial load and increase risk of variation in laboratory performance and result[35]. Contaminated samples are more likely at late stages of treatment, a time when patients' bacillary load has fallen significantly and they have greater difficulty producing quality sputum. In our data we show the impact of this effect with significant loss of TTP data points particularly at late stages of treatment. This further compromises the utility of culture to inform clinical decision at later timepoints. TB-MBLA is not affected by contamination and produced a quantitative result for the *M. tuberculosis* viable count at both early and late stages of treatment.

This study has highlighted several strong attributes of TB-MBLA but there are limitations that require more studies to address. A study evaluating many more patients with resistant disease that are more likely to respond poorly to therapy is needed to establish a marker for poor response early in treatment. For example, how much bacillary load the patient should clear in the first days of treatment in order to be regarded as good or poor responder? Secondly, what is the effective testing frequency that usefully maps patient's treatment response and their clinical outcomes? More optimisation studies to explain discordant results between culture and TB-MBLA and to simplify the assay so that it is easy to perform with minimal hands on time and available at affordable cost.

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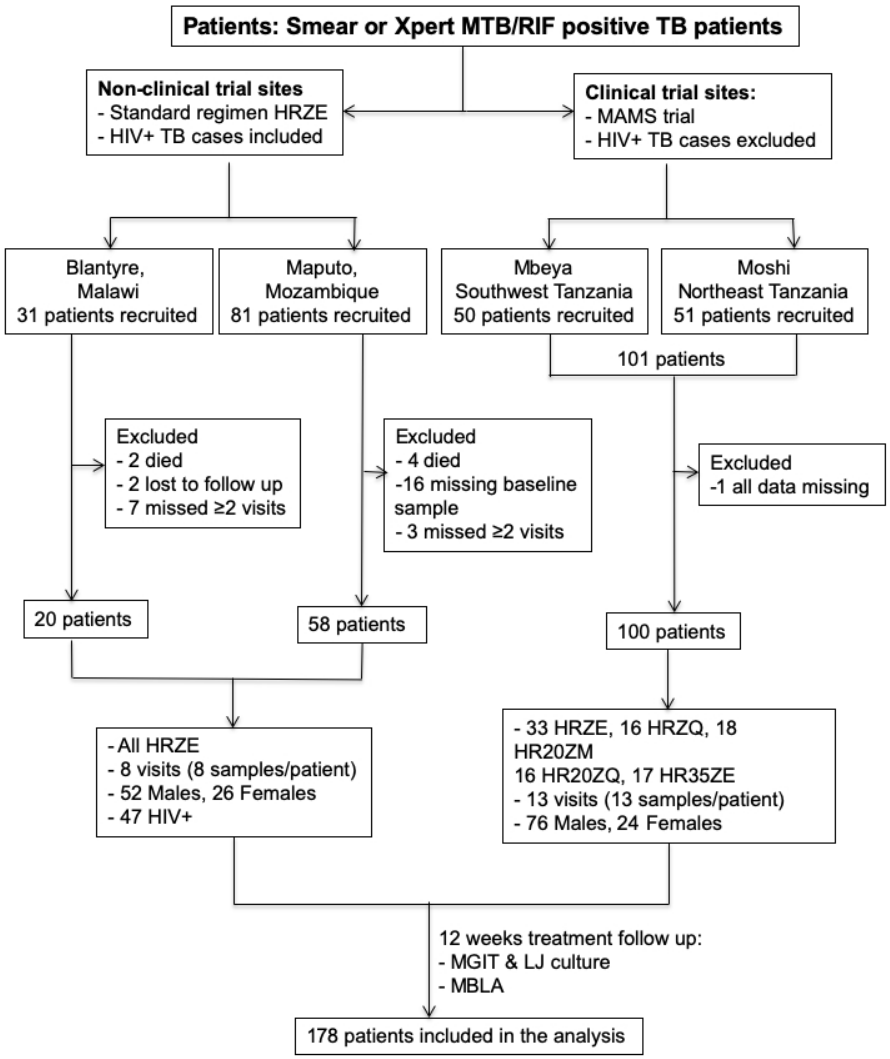
Figure legends

Supplementary figure 1. The study flow diagram. Patients who died earlier on in the study, lost to follow-up, missing baseline sample, missing ≥2 treatment visit results and/or had no data at all were excluded from the analysis, leaving 178 patients.

Supplementary figure 2. The reproducibility of TB-MBLA in different laboratory settings. A) the results of high BCG panel (n=24 per site), B) results of low concentration BCG panel (n=24 per site), and C) the extraction (internal control retrieved from patient samples as marker of extraction efficiency (n=1633 across the 4 sites). Each point represents an extracted sample and the red line is the median of the log eCFU/ml of the spiked extraction control.

Supplementary figure 3. The concordance and discordance of MGIT culture and TB-MBLA results. Orange and green bars are cases with definitive MBLA positive or negative result but indeterminate MGIT culture result due to contamination that grew in number in late stages of treatment.



Study flow diagram: Patient recruitment & Inclusion in analysis

Study flow chart

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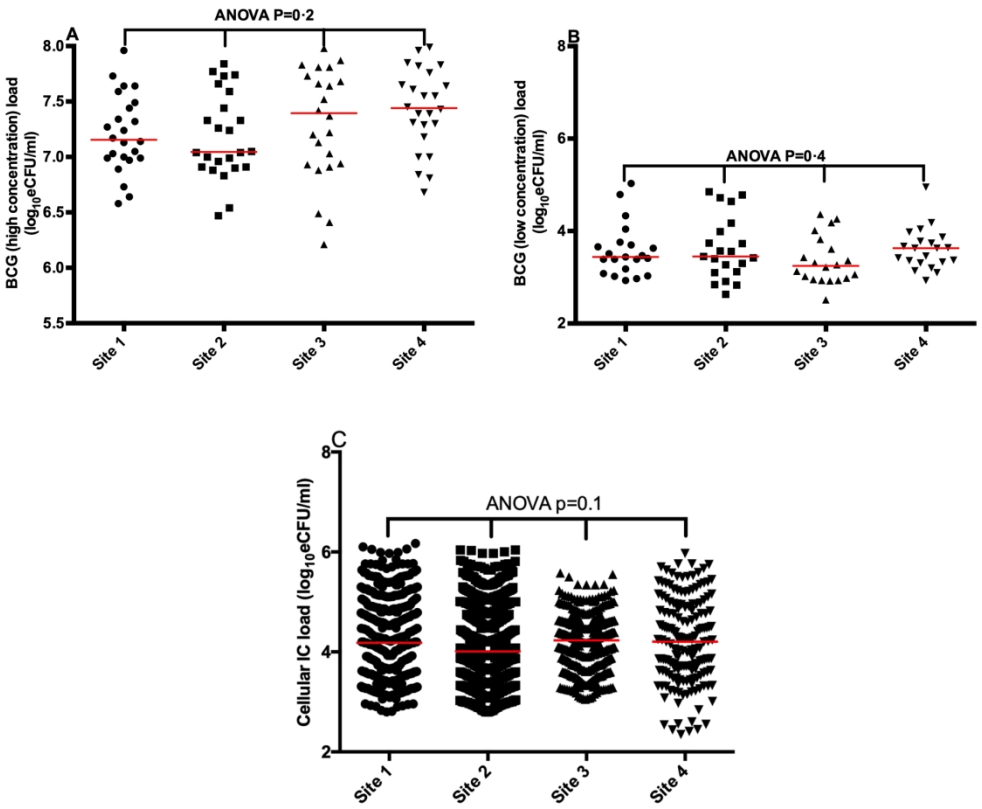

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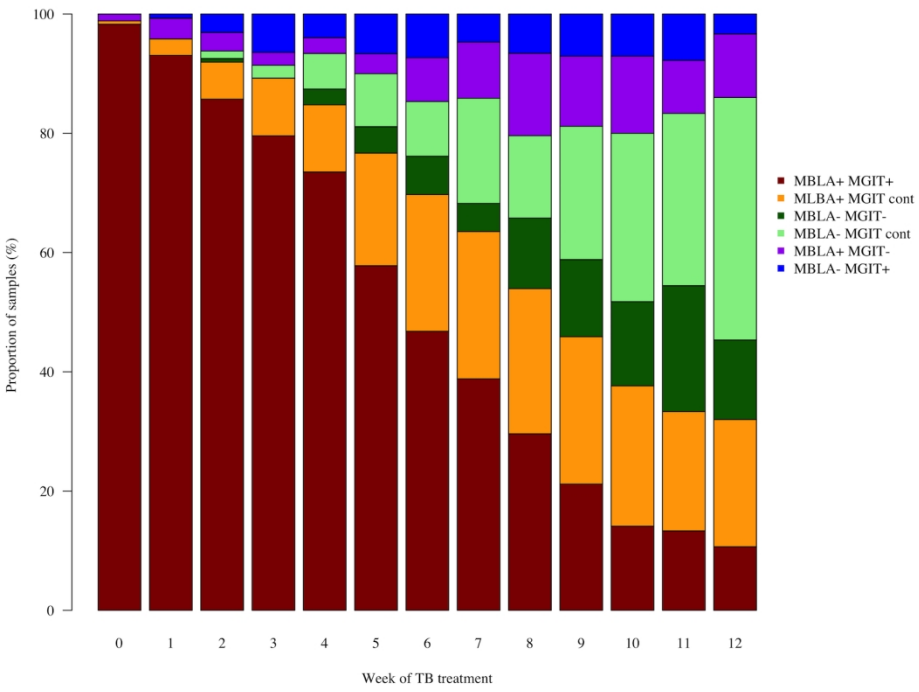

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